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Interaction with the Yes-associated protein (YAP) allows TEAD1 to positively regulate NAIP expression

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ABSTRACT

Although the expression of the neuronal apoptosis inhibitory protein (NAIP) gene is considered involved in apoptosis suppression as well as in inflammatory response, the molecular basis of the NAIP gene expression is poorly understood. Here we show that the TEA domain protein 1 (TEAD1) is able to positively activate the transcription of NAIP. We further demonstrate that this regulation is mediated by the presence of the endogenous Yes associated protein (YAP) cofactor, and requires the interaction with YAP. We finally identified an intronic region of the NAIP gene responding to TEAD1/YAP activity, suggesting that regulation of NAIP by TEAD1/YAP is at the transcriptional level.

1. Introduction

The neuronal apoptosis inhibitory protein (NAIP), the first member of the inhibitor of apoptosis proteins (IAPs) family, was shown to be absent in patients with severe forms of spinal muscular atrophy (SMA) [1]. Further studies revealed that this protein renders cells resistant to apoptosis under both in vivo and in vitro conditions [2–4]. NAIP, a 160 kDa protein, has three regions of baculoviral inhibitor of apoptosis repeat (BIR) domains, a nucleotide binding oligomerization domain (NOD), and a leucine rich repeat (LRR) motif. Anti apoptotic function of NAIP is attributed to its BIR3 domain that inhibits caspase-9 and to its BIR2 domain that inhibits caspase-3 [5,6]. Nevertheless, thanks to the presence of the NOD domain and LRR motif, which is unique among the IAPs, it has been suggested that NAIP functions are distinct from these of other IAP proteins by harbouring these domains [7], and it has been proposed to act as modulator in assembling the "inflammasome", a cytoplasmic protein complex, for the activation of inflammatory caspases in response to several stimuli [8,9]. These different biological functions proposed for NAIP could be taken into account by different regulation mechanisms, based, for example, on different spliced variants, as described [10], and reported on the NCBI site (provided by RefSeq, Jul 2008).

We recently showed that alteration of TEA domain protein 1 (TEAD1) expression levels confers apoptotic resistance in HeLa cells, through transcriptional up-regulation of another member of the IAP family, Livin [11]. In that same paper we also show that NAIP is up- and down-regulated according to similar variation of TEAD1 expression levels, suggesting a positive regulation of NAIP by TEAD1. TEAD1 belongs to the family of conserved eukaryotic transcription factors (TEAD proteins), characterized by the TEA/ATTS DNA binding domain [12–14], that, in mammals, includes four closely related *Tead* genes (*Tead1* to *Tead4*) [15,16]. The transcriptional activator function of the TEAD proteins appears to require their interaction with coactivator proteins [17]. One of the well characterized, in vitro and in vivo, cofactor of mammalian TEAD proteins is the Yes-associated protein (YAP) [18–21]. YAP is the effector of the Hippo tumour suppressor pathway that restricts organ growth by keeping in check cell proliferation and promoting apoptosis in mammals and also in *Drosophila* [22,23].

To gain insights on the transcriptional regulation of NAIP, based on our previous data [11], we decided to study the role of the transcription factor TEAD1 and its relationship with YAP and the

Abbreviations: TEAD, TEA domain; IAP, inhibitor of apoptosis protein; NAIP, neuronal apoptosis-inhibitory protein; YAP, Yes-associated protein; Yki, Yorkie; LATS, large tumour suppressor; MST, mammalian Ste20-like protein kinase

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components of the Hippo pathway in HeLa cells. Using a TEAD1-inducible cellular Tet-On system we show a positive TEAD1-dependent induction of the expression of the NAIP gene. Moreover, we show that this induction needs the presence of endogenous YAP as well as the interaction with this transcriptional co-factor, and that the Hippo pathway could interfere with this regulation. We finally identify several intronic regions of NAIP containing putative DNA binding motifs for TEAD proteins. Interestingly we show that one of these regions, intron 11, contains a TEAD1 tandem *cis*-regulatory binding element and responds to TEAD1/YAP transcriptional activity suggesting that regulation of NAIP by TEAD1/YAP is at the transcriptional level.

2. Materials and methods

2.1. RNA extraction and quantitative RT-PCR

Total RNA extraction, cDNA synthesis and real-time PCR were performed as described previously [24]. Amplification of the *GAPDH* and *RPL13* genes were performed to ensure comparability between cDNAs from different samples. Primers and annealing temperatures for *GAPDH*, *RPL13*, *TEAD1*, *YAP* and *NAIP* amplification were reported in [11]. For each gene, values were averaged over at least three independent measurements. Three independent RNA isolation experiments were performed for all experiments, and means were calculated. To estimate the significance of the differences between means, the parametric statistical Student's *t*-test was used.

2.2. Cells, plasmids and transfection conditions

HeLa and HeLa T-Rex (Invitrogen), MCF7 and BUA cells growth conditions and the following plasmids (TEAD1, TEAD1-Y421H, TEAD1 D55-121, YAP and TEAD-VP16) were previously described [11]. 10^6 cells were transfected with a total amount of 2400 ng of DNA. Cells were transfected either with 2400 ng of pXJ40 empty vector (controls) or with equal amounts of indicated expressing plasmids (800 ng per construct). The total amount of transfected DNA was kept constant by addition of empty vector (pXJ-40) wherever necessary. MST2 and LAST1 cDNAs (gifts from X. Yang) were subcloned into the pcDNA3.1 vector to allow expression of these cDNAs under the control of a CMV promoter. The genomic region of intron 11 of NAIP was amplified by PCR (forward primer 5′ CCGCTCGAGCGGTCCTTAGAAGCACCAATCAGT reverse primer CCGCTCGAGCGGCCAGATCGGAAGAGATATC) and subsequently cloned into pLG3-TK (gift of A. Payne). A 53 bp fragment from the promoter of the 3β-hydroxysteroid dehydrogenase-isomerase (3βHSD) gene was cloned into the pGL3-TK plasmid. This 53 bp region of the 3βHSD promoter contains binding sites for the TEAD factor. Luciferase assays were performed as described previously [11]. All experiments were performed at least three times.

2.3. Tet-On TEAD1 clones

To obtain inducible clones expressing TEAD1, we used the T-REX Tetracycline-Regulated Expression System for Mammalian Cells (Invitrogen), according to the manufacturer's instruction. Briefly, the HeLa T-Rex cell line was transfected with pcDNA 4/TO in which the TEAD1 cDNA was cloned. Selection of resistant clones was performed with an optimal dose of antibiotic (400 μg/ml of Zeocin), assessed during a previous toxicity assay. Following amplification, the clones were treated with 1 μg/ml of doxicycline to assess the induction of TEAD1. Seven independent clones were found positive to induction, and 2 were chosen for subsequent experiments.

2.4. siRNA

siRNA against *YAP* or negative-control RNA were chemically synthesized (Dharmacon Research, Lafayette, USA). Synthetic siRNAs were transfected with TransIT-TKO Transfection Reagent (Euromedex) according to the manufacturer's instructions.

2.5. Western blot analyses

Western blot analyses were performed as described previously [11].

2.6. ChIP experiments

Subconfluent cells grown in five 150-mm plates were fixed in situ with 1% Formaldehyde for 10 min at room temperature, then fixation was blocked with 125 mM glycine, cells were rinsed and scrapped in PBS. Cells were first lysed in Lysis buffer A (50 mM Hepes pH 7.5, NaCl 140 mM, EDTA 1 mM, glycerol 10% NP-40 0.5% and Triton X-100 0.25%), then centrifugated and rinsed in lysis buffer B (Tris 10 mM pH 7.5, NaCl 200 mM, EDTA 1 mM, EGTA 0.5 mM), centrifugated again and finally resuspended in lysis buffer C (Tris 10 mM pH 7.5, NaCl 100 mM, EDTA 1 mM, EGTA 0.5 mM, Na–deoxycholate 0.5%, *N*-lauryl sarcosine 0.5%). Chromatin was sheared in a Bioruptor sonicator, used at high power with 30/30 s cycles for 20 min, then 1% Triton X-100 was added to lysates, and cell debris were centrifugated. In parallel, 30 μg anti-YAP or anti-TEF1 were incubated with 200 μl ProteinG Dynabeads for 4 h in PBS containing 0.1% BSA. Lysates were incubated with the beads overnight, beads were then washed five times in wash buffer (50 mM Hepes pH 7.6, 500 mM LiCl, 1 mM EDTA, 0.7% Na–deoxycholate, 1% NP-40) and once in TBS. DNA–protein complexes were then eluted in 10 mM Tris pH 8, 1 mM EDTA, 1% SDS at 65 °C for 15 min. Crosslinked was reversed at 65 °C overnight, then recovered DNA was treated with RNase, proteinase K and purified using phenol–chloroform extraction. qPCR analysis was then carried on using Input samples subjected to the same procedure as controls. Primers for Q-PCR are listed in Table S1B.

2.7. Electrophoretic mobility shift assay (EMSA)

The sequences of double-stranded oligonucleotides end-labeled with biotin (Sigma) are listed in Table S1C. EMSA analysis was performed according to the Thermo Scientific LightShift Chemiluminescent EMSA Kit.

3. Results

3.1. TEAD1 positively regulates NAIP expression and depends on endogenous YAP expression

Very little is known about the regulation of the NAIP gene that could be explained, at least partially, by the extreme repetitiveness and complexity of the genomic organisation of the region containing the gene in humans, as previously reported [25].

For this reason, to gain insight on the regulation of NAIP, we took advantage of our previously published data [11] where we showed that overexpression or inhibition of TEAD1 expression in HeLa cells resulted in a increase or decrease in the level of endogenous NAIP mRNA, suggesting a positive regulation of NAIP by TEAD1. Since the overexpression experiment was performed through transfection of high amounts of TEAD1 plasmid, we decided to perform Tet-On clones with the T-REx™ HeLa cell lines (Invitrogen), allowing a more physiologically and well monitored induction of TEAD1 expression levels. Namely, using this technique

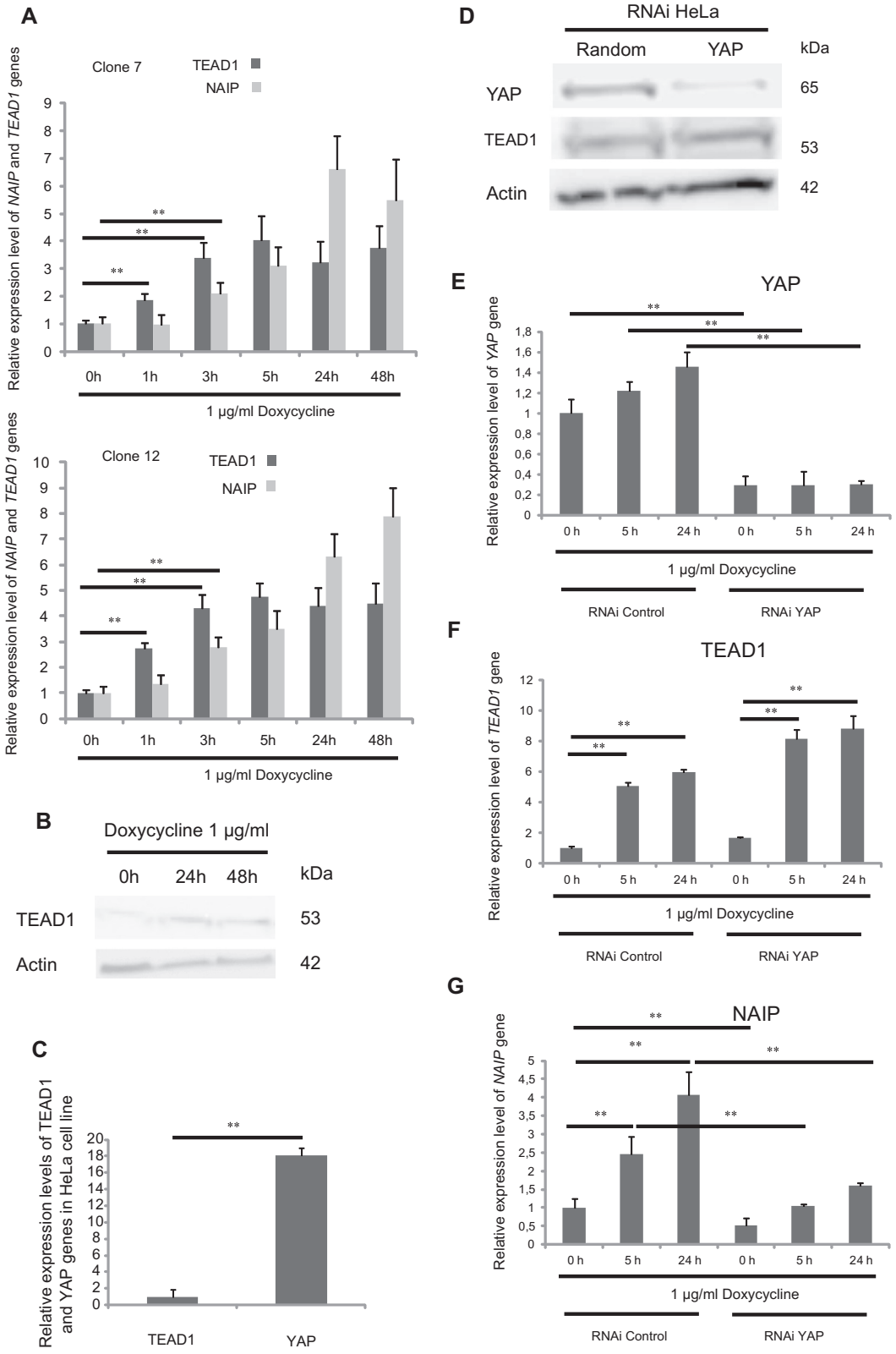

Fig. 1. TEAD1 positive regulation of NAIP expression depend on endogenous YAP expression. (A) RT-Q-PCR quantifications for the transcripts of the *TEAD1* and *NAIP* genes were performed on 10^5 T-REx™ HeLa cells (clone 7 and 12), in the presence (form 1 h to 48 h) or absence of 1 µg/ml of Doxicycline. *TEAD1* and *NAIP* mRNA quantification were normalized to endogenous GAPDH mRNAs for internal control. Values obtained for control transfection are normalized to 1 for each gene. Histograms display mean values from a minimum of three independent replicates. Error bars indicate S.D. Asterisks indicates statistical significance. The *P* values were calculated by the Student's *t*-test from three independent experiments. $^{**}P < 0.01$. (B) Western blot analysis of lysates from HeLa cells treated with 1 µg/ml of doxicycline for 24 h and 48 h (clone 7). TEAD1 and actin levels are presented. (C) Relative mRNA levels for *TEAD1* and *YAP* genes in HeLa cells. GAPDH served as internal control to normalize the *TEAD1* and *YAP* mRNAs. *TEAD1* levels are set to 1. $^{**}P < 0.01$, *t*-test. (D) Western blot analysis of lysates from HeLa cells treated with siRNA (random or YAP). TEAD1, YAP and actin levels are revealed. (E–G) RT-Q-PCR quantifications for the transcripts of the *YAP*, *TEAD1* and *NAIP* genes were performed on T-REx™ HeLa cells (clone 7) in the presence (for 5 or 24 h) or absence of doxicycline and treated with 10 nM of random or YAP-specific siRNA.

it is possible to induce, in a time-dependent fashion, the expression of TEAD1 by adding the antibiotic doxicycline. We obtained several positive inducible clones, and we decided to focus our study on two representative clones (7 and 12). We observed in both clones (Fig. 1A) a rapid induction of the mRNA (measured by RT-Q-PCR) of TEAD1 after 1 h induction, with a maximum obtained at 5 h. Western blot analysis revealed 24 and 48 h after induction, an increase also in the level of the TEAD1 protein in treated cells versus control cells (Fig. 1B). Interestingly, this induction is well correlated with an increase of NAIP mRNA, starting at 3 h of treatment (Fig. 1A). It is worth stressing that this induction is increased after 24 h of induction, according to the observed increase TEAD1 levels. This result, together with our previous data [11] showing that a decrease of TEAD1 by siRNA correlates positively with a significant decrease (65–70%) of NAIP mRNA, suggests that expression of NAIP can be modulated positively by TEAD1.

However, it is well established that TEAD1 must interact with cofactors to activate transcription [26,27]. The fact that expression of NAIP is rapidly induced (3 h) after TEAD1 induction, suggests that this cofactor must still be present, and in sufficient amount, in the cells when doxicycline is added. Since one of the main cofactors of TEAD1 is the transcriptional co-activator YAP [18,20], and since RT-Q-PCR analysis of endogenous YAP expression in HeLa cells showed very high levels (20-fold) of mRNA when compared to TEAD1 expression (Fig. 1C), we focused our attention on YAP for further analyses. To asses if YAP expression is necessary for TEAD1 to induce NAIP expression, we examined the effect of a decrease of mRNA of YAP on TEAD1-dependent NAIP induction. Specific mRNA knockdown by a combination of four independent synthetic siRNAs reduced YAP mRNA by 75–80% in clone 7 (Fig. 1E) and in clone 12 (data not shown). Western blot analysis showed also a reduction at the protein level of YAP (Fig. 1D), while the endogenous level of TEAD1 remains unchanged (Fig. 1D). When treated with doxicycline, HeLa clones showed, as expected, a time-dependent increase of TEAD1 expression (Fig. 1F), and this independently of the endogenous YAP levels. Moreover, we observed that without induction of TEAD1 (point 0 h), the basal expression of NAIP is reduced when YAP is knocked-down (Fig. 1G), suggesting that endogenous YAP is required for NAIP expression. To further confirm this observation, we also knocked down YAP protein in another cellular model, the BUA cells (a human fibroblast cell line), whose levels of endogenous YAP are higher when compared to HeLa cells (Fig. S1A). Interestingly, a decrease of mRNA and protein levels of endogenous YAP (Fig. S1B and C) is correlated to a significant decrease of endogenous NAIP mRNA level (Fig. S1D), according with results observed in HeLa cell line.

Finally, when HeLa cells were treated with doxicycline, the induction of NAIP expression was completely abolished, when YAP was knocked-down (Fig 1G). Taken together, these results indicate that the presence of endogenous YAP is required for basal expression as well as TEAD1-dependent induction of NAIP expression.

3.2. Interaction of TEAD1 with YAP is required for NAIP activation and is affected by the Hippo pathways

The biochemical nature of the YAP–TEAD interaction, that is crucial to mediate YAP-induced gene expression, has been recently reported [28,29]. To investigate whether YAP/TEAD1 interaction is involved in NAIP up-regulation by TEAD1, two strategies were used. In one approach, TEAD1 was overexpressed with YAP in HeLa cells. Western blot analysis of lysates from HeLa cells transfected with a void plasmid (control), TEAD1 and/or YAP were performed, and TEAD1, YAP and actin levels were revealed. As shown in

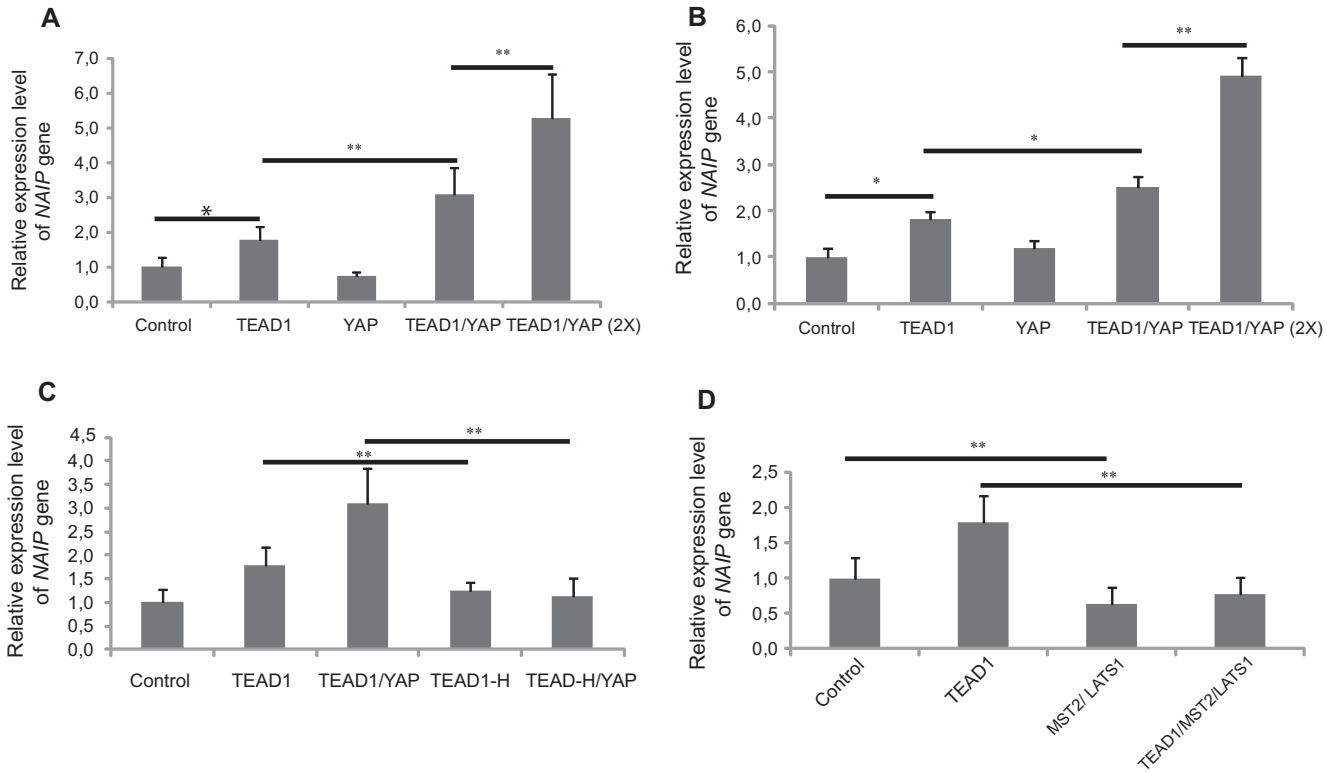

Fig. 2. Interaction of TEAD1 with YAP is required for NAIP activation and is affected by the Hippo pathways. RT-Q-PCRs for *NAIP* mRNAs from HeLa (A, C and D) and MCF7 cells (B) transfected with 800 ng of the pXJ40 empty plasmid (control), TEAD1 or YAP-expressing plasmid (A and B) TEAD1-H (C) and MST2/LATS1 expressing plasmids (D), alone or in combination. mRNA quantification was normalized to endogenous *GAPDH* mRNAs for the internal control. Mean ± S.D. calculated from three independent replicates are displayed. $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test.

Fig. S1E, increased levels of TEAD1 in transfected cells with TEAD1 alone or with YAP, versus control cells transfected with a void plasmid are observed. Similar results were obtained with YAP or YAP/TEAD1 transfected cells (Fig. S1E). As previously published [11], we observed a slight, but significant, increase in NAIP expression, when TEAD1 was overexpressed (Fig. 2A). When YAP was overexpressed alone, no effect on NAIP induction was observed (Fig. 2A). On the contrary, when TEAD1 and YAP were co-expressed, TEAD1-dependent NAIP induction was increased in a dose dependent manner (Fig. 2A), indicating a cooperative effect of these two proteins on NAIP expression. In order to confirm this observation we overexpressed TEAD1 and/or YAP in the MCF7 cell line (a cell line derived from Human mammary tumors), whose levels of endogenous TEAD1 and YAP proteins are very low when compared to HeLa cells (Fig. S1A). Consistent results were obtained after overexpression of TEAD1, with a slight, but significant induction of NAIP (Fig. 2B). Interestingly when both TEAD1 and YAP were co-expressed, TEAD1-dependent NAIP induction was increased (Fig. 2B), indicating a cooperative effect of these two proteins on NAIP expression, as observed in HeLa cells. In the second approach, we analyzed in parallel how a mutant TEAD1 protein unable to interact with YAP affects NAIP induction. Advantage was taken of the mutation of a highly conserved tyrosine in the YAP-binding domain of TEAD1, (TEAD1–Y421H, hereafter referred to as TEAD1-H) (Fig. 3A). This mutation causes the human genetic disease known as Sveinsson's chorioretinal atrophy [30], and strongly reduces both YAP/TEAD1 interaction and activity [28]. Contrary to wild-type TEAD1, TEAD1-H was unable to activate the expression of a TEAD1 responsive reporter when transfected with YAP, as previously published [11] confirming that interaction with YAP is severely impaired. HeLa cells transfected with the mutant forms of TEAD1 showed no induction of NAIP expression levels (Fig. 2C), suggesting that interaction with endogenous YAP is required for NAIP induction. According to this result, the cooperative effects observed with YAP and TEAD1 on NAIP induction were prevented with TEAD1-H (Fig. 2C) indicating that they rely on the TEAD1/YAP interaction. Altogether, the results obtained with transfection of YAP and TEAD-H, which is unable to interact with YAP, clearly indicate that the TEAD1-dependent NAIP up-regulation requires the interaction with YAP.

It is well established that YAP activity is regulated through its shuttling between the nucleus and the cytoplasm, under the control of the Hippo pathway. Indeed, activation of the Hippo pathway leads to YAP phosphorylation, by the large tumour suppressor-1 (LATS1) kinase, and its exclusion from the nucleus. [31–33]. To investigate whether the Hippo pathway is involved in NAIP up-regulation by affecting TEAD1/YAP interaction, we overexpressed TEAD1 with both core components of the Hippo pathway, mammalian Ste20-like-2 (MST2) and LATS1 kinases. When MST2 and LATS1 were overexpressed, a slight but significant decrease of NAIP mRNA was observed (Fig. 2D). This results is consistent with the fact that endogenous YAP is required for TEAD1-dependent NAIP induction, as observed when YAP is knocked-down (Fig. 1C). Interestingly, when MST2 and LATS1 were co-expressed with TEAD1, NAIP induction was completely abolished, with a significant decrease of its expression, compared to TEAD1 (Fig. 2D), suggesting that an active and nuclear form of endogenous YAP is required for the TEAD1-dependent NAIP induction.

3.3. TEAD1 transcriptionally regulates NAIP expression

Consistent with our observations, there has been speculation that the expression of NAIP may be directly regulated by TEAD1. The human NAIP locus (NCBI Reference Sequence No. NG_008724.1) was therefore examined for putative TEAD binding sites using the Cis-element Cluster Finder (CISTER) program (http://zlab.bu.edu/~mfrith/cister.shtml). As positive control, we checked the promoter of the human chorionic somatomammotropin (hCS) gene that is known to respond to the TEAD transcription factor [34]. As expected, analysis of this sequence for TEAD binding sites showed several, highly significant binding sites in this region (data not shown). Initial screening of the NAIP genomic region with the CISTER program revealed five significantly TEAD-rich regions within introns 3 (713 bp), 9 (1528 bp), 11 (705 bp) and 12 (340 bp), as well as in exon 9 (1312 bp) (Fig. 4A). Attention was focused on intronic regions. Of the four introns, intron 11 was selected for further analyses, because it showed a higher number of putative sites (27) (Table S1A) compared to introns 3 and 12, and because the length of the genomic region is shorter compared to intron 9. The genomic fragment, corresponding to the intron 11 TEAD-rich region of NAIP was amplified by PCR and cloned into the pGL3-TK plasmid, in order to obtain a luciferase reporter construct (Int.11). As positive control, we cloned in the same vector a 53 bp fragment from the promoter of the 3β-hydroxysteroid

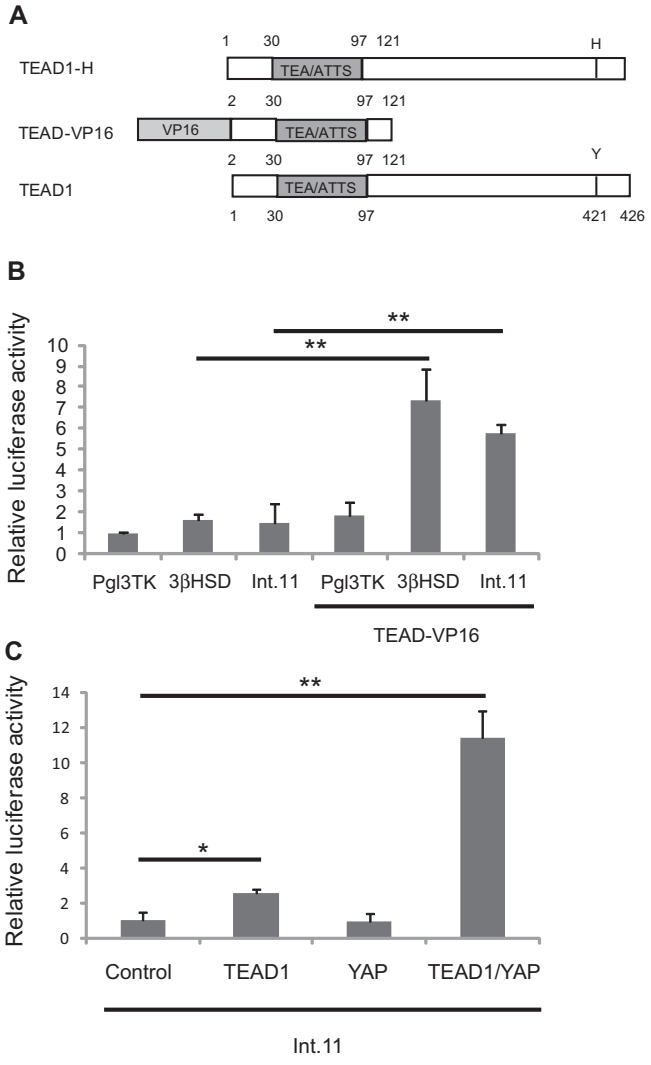

Fig. 3. TEAD1 transcriptionally regulate NAIP expression. (A) Structural features of TEAD1, the TEAD1-H mutated plasmid and structure of the TEAD-VP16 plasmid. (B and C) Relative luciferase activity assessed after transfection of 8×10^4 HeLa cells with the pGL3-TK, intron 11 and 3βHSD luciferase reporters plasmids alone or with 200 ng of the TEAD-VP16, TEAD1 and YAP plasmid. Normalization of transfection efficiency was obtained by co-transfection of a β-galactosidase-expressing vector and assaying of β-gal activity in cell extracts. Error bars indicate standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

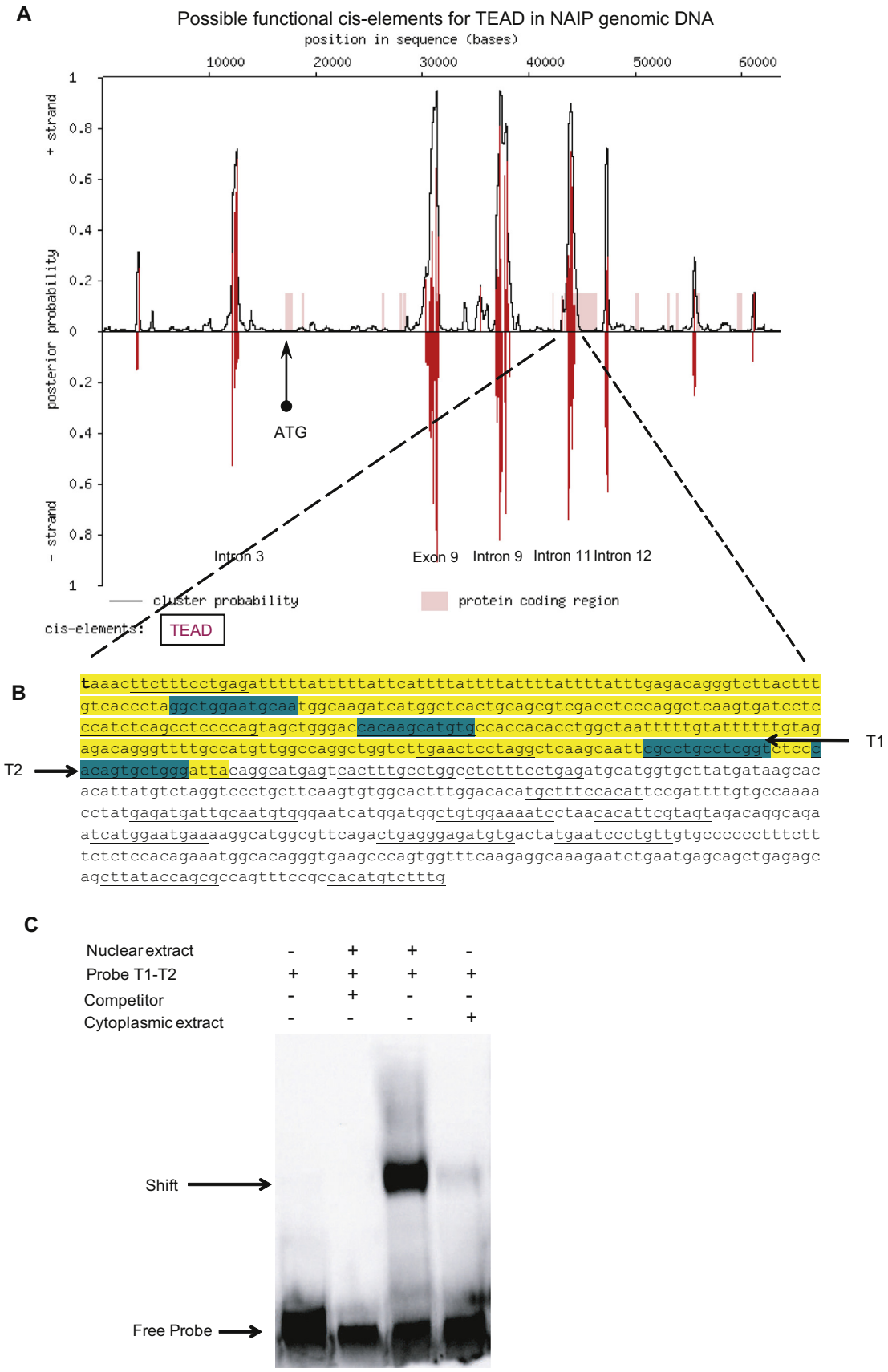

Fig. 4. Possible functional *cis*-elements for TEAD in NAIP genomic DNA. (A) Possible functional *cis*-elements (in red) for TEAD in NAIP genomic DNA are assessed by using the Cis-element Cluster Finder (Cister) program. Positions of starting codon (ATG) and intron/exon regions are indicated. (B) Alu sequence of about 300 bp, in yellow, in the intron 11 sequence is showed. The 27 possible functional *cis*-elements for TEAD in the intron 11 sequence are underlined. The four *cis*-elements with the higher probability are showed in green. (C) EMSA using nuclear extracts (8 μg proteins) obtained from HeLa cells transfected with TEAD1 plasmid. Controls include: BSA (lane 1), competitor (lane 2) and cytoplasmic extract (lane 4).

dehydrogenase-isomerase (3βHSD). This 53 bp promoter region contains binding sites for TEAD factors [35].

To investigate whether TEAD1-dependent NAIP induction is mediated through the transcriptional activity of TEAD1, a constitutively active form was used [11] where its YAP binding domain [19] is replaced by the activation domain (AD) of VP16 (TEAD-VP16) (Fig. 3A). When this constitutively active form was co-tranfected with the native plasmid pGL3-TK, no induction was observed compared to plasmid pGL3-TK alone (Fig. 3B). Conversely, as expected, the TEAD-VP16 fusion protein highly stimulated the TEAD-responsive reporter construct, 3βHSD (Fig. 3B). Interestingly, overexpression of TEAD-VP16 with the NAIP-Intron11 containing plasmid resulted in a 6-fold reporter activation, compared to the control (Fig. 3B). To confirm this results we transfected HeLa cell line with the NAIP-intron 11 luciferase construct, together with TEAD1 and/or YAP. According to results obtained with endogenous NAIP mRNA, we observed a 2.5fold induction of luciferase activity with TEAD1 alone and no significant effect with YAP. Most importantly, a high increase (10-fold) in luciferase activity was observed when TEAD1 and YAP are co-expressed (Fig. 3C). These results confirmed the results observed with TEAD-VP16, and strongly suggest, in agreement with the endogenous NAIP induction experiments, that TEAD1 and YAP regulate transcription of NAIP rather than its mRNA stability.

The intron 11 of NAIP contains 27 possible functional *cis*-elements for TEAD (Fig. 4B and Table S1A). The presence of an Alu repeated sequence (of about 300 bp) at the 5′ of the intron (Fig. 4B) impeded the analysis of this region by using Chip experiments. We therefore tried to identify TEAD binding sites in the 3′ region of the intron 11. For Chip experiments the promoter of the *CTGF* gene was chosen as a positive control. Q-PCR results showed, as previously published [18], a specific chromatin binding site for TEAD1 and YAP proteins (data not shown) in *CTGF* promoter. Chip experiments performed with the 3′ region of the intron 11 did not reveal any specific chromatin binding for TEAD1 protein. However, since the 4 putative binding sites with the highest probability (more than 0.60) (Table S1A) are located in the non-analyzable Alu repeated sequence (Fig. 4B) we carried out electrophoretic mobility shift assays (EMSA). Of the four sites, attention was focused on the two sites located in tandem at the 3′ end of the Alu region (Fig. 4B, probes T1 and T2), since it has been reported that the DNA binding affinity of TEAD proteins is higher with repeated tandem binding sites [36]. EMSA was employed using custom oligonucleotide probes spanning T1 and/or T2 putative sites (Table S1C), and nuclear extracts from HeLa cells transfected with control plasmid, TEAD1 or TEAD1 D55-121, a TEAD1 protein deleted of its TEA domain [11]. Cytoplasmic extract and competition assays were performed to confirm the specificity of the TEAD1–DNA complexes (Fig. 4C). Nuclear extracts from cells transfected with TEAD1 and oligonucleotide specific for the promoter of the *CTGF* gene were used as a robust positive control and revealed a retardation of the probe due to DNA/TEAD1 complex (data not shown). Interestingly, when EMSA experiments were performed with nuclear extracts from TEAD1 overexpressing cells, we observed a retardation of the probe with oligonucleotides spanning TEAD1-like site T1–T2 (Fig. 4C) due to the formation of DNA/TEAD1 complexes, but not with oligonucleotides spanning only the T1 or T2 sites (data not shown). As expected, TEAD1 DNA-binding activity in nuclear extracts from control or TEAD1 D55-121 was not observed with T1, T2 and T1–T2 oligonucleotides (data not shown). Taken together, TEAD1 complexes binding on these TEAD1-like tandem sites T1–T2 suggest that the expression of NAIP may be transcriptionally regulated by TEAD1 binding to these sites located within this intronic region in the gene locus.

4. Discussion

Besides the well described anti-apoptotic functions of NAIP [2–4], several studies propose NAIP as a regulator for inflammasome formation that activates inflammatory caspases in response to different stimuli [8,9]. This process, well studied in mice, is also known as "pyroptosis", that differs from apoptosis by the specific activation of caspase-1 in response to intracytoplasmic pathogen-associated molecular patterns by NOD-like receptors (NLR) leading to the formation of a large pyroptosome (or inflammosome) in macrophages [37]. Interestingly, a recent study showed that also human NAIP is able to promote caspase-1 activation and subsequent pyroptosis in response infection in macrophage [38]. Since the different domains of NAIP seem to be involved in different functions, the existence of splicing variants described for its gene could, at least partially, explain these different functions [10]. However, the events regulating NAIP transcription are largely unknown.

Here we demonstrate, for the first time, a positive regulation of NAIP by TEAD1 which requires the interaction of TEAD1 with the YAP cofactor, and this could be affected by the core components of the Hippo pathway. Interestingly we identified a novel region in the intron 11 responding to TEAD1/YAP activity, and to TEAD1/YAP overexpression that could act as enhancer of NAIP expression, as well as other putative regions in introns 3, 9 and 12. It is well known that enhancer sequences can also be found within introns. In particular a recent study showed a possible link between NF-κB activity and expression of NAIP potentially involving functional NF-κB binding sites in the promoter and in the second intron of the NAIP gene [39]. In the present study, the application of EMSA reveals TEAD1 DNA-binding to two previously uncharacterized TEAD1 tandem regulatory binding elements in the NAIP intron 11 regions, suggesting that TEAD1 can regulate the transcription on the NAIP gene through *cis*-regulatory elements that resemble the TEAD consensus binding motif.

Moreover, the consequences of TEAD1 and/or YAP overexpression on basal apoptosis in HeLa cells were investigated. As showed in Fig. S1G, in TEAD1 overexpressing cells apoptosis was slightly reduced, while NAIP expression is slightly induced (Fig. S1F). Interestingly, when TEAD1 and YAP were co-transfected, a significant decrease of apoptosis was observed, according with the up-regulation of NAIP mRNA levels (Fig. S1F and G). Taken together, these results suggest a possible anti-apoptotic function of NAIP in HeLa cells. However further study are needed to prove the effective role of NAIP in apoptosis protection in HeLa cells.

At the present time, the biological significance of our observations is necessarily speculative. However, since our data strongly suggest that interaction of TEAD1 with YAP is required for NAIP up-regulation, it will be interesting to investigate the physiological role of this regulation in tissues where these proteins are expressed. Interesting potential candidates include mammalian placenta, since it has been showed that NAIP is highly expressed in human decidual cells of the decidua basalis, the maternal portion of the placenta [40]. Importantly TEAD proteins as well as their most studied cofactors YAP and the Vestigial-like proteins (Vgll1-4), the mammalians homologues of the *Drosophila* transcriptional co-activator Vestigial (Vg), are also expressed in the cells precursor of the placenta or in mature placenta [41–43]. To better understand the biological relevance of this new regulation, in physiological or pathological conditions, as well as to assess a possible direct link between the TEAD1/YAP transcription factor and the identified intronic regions, further analyses are required to gain insight into the physiological significance of this new regulation of NAIP by the TEAD1/YAP transcription factor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.013.

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